Reference values for B-cell surface markers and co-receptors associated with primary immune deficiencies in healthy Turkish children

**Elif Azarsiz, Neslihan Edeer Karaca, Guzide Aksu
and Necil Kutukculer**

Abstract

In order to evaluate B-lymphocyte subsets of patients with primary immunodeficiencies, the normal values for national healthy children have to be used as a reference. Recently, B-cell co-receptor markers (CD19, CD21, and CD81) and CD20, CD22, and CD27 deficiencies have been reported in relation with different primary immunodeficiency diseases. The objective of this study was to establish national reference values for B-lymphocyte co-receptors and some surface markers, CD20, CD22, CD27, as well as classic lymphocyte subsets in the peripheral blood of healthy children. A total of 90 healthy children were included in this study. Complete blood counts were performed and cells with CD3, CD4, CD8, CD19, CD16/56, CD20, CD21, CD22, CD27, and CD81 surface markers were simultaneously detected by flow cytometry. The children were evaluated in three age subgroups, 0–1, 1–6, and >6 years, and minimum, maximum, mean, mean minus standard deviation, and 2.5–97.5 percentile values were all determined. By establishing reliable reference ranges for these surface markers, we hoped to help identifying and classifying some primary immunodeficiency patients, especially those defined as unclassified hypogammaglobulinemia and those without definite diagnosis.

Introduction

B cells play a central role in humoral immunity preventing infections caused by extracellular infectious pathogens. B-cell development starts with maturation in the bone marrow followed by antigen-driven differentiation in peripheral lymphoid organs and shows different stages of maturation during the course of life especially in the first 5 years.[1] Several B-cell deficiencies result from abnormalities of B-cell development.[2]

B cells have adaptive and variable transmembrane receptor proteins located on the outer surface which are called as "B-cell receptor (BCR)" that binds antigens. This binding causes B-cell activation, proliferation, and differentiation to generate a population of antibody-secreting plasma B cells or memory B cells. B cells also possess a co-receptor complex composed of CD19/CD21/tetraspan protein Tapa-1 (CD81) which can modulate BCR signal transduction. CD21 binds C3d opsonized antigenic particles, such as bacteria or enveloped viruses. C3d binds

Department of Pediatric Immunology, Faculty of Medicine, Ege University, Izmir, Turkey

Corresponding author:
Elif Azarsiz, Department of Pediatric Immunology, Ege University School of Medicine, Izmir, Turkey.
Email: elif.azarsiz@ege.edu.tr

to CD21 aggregating the co-receptor complex with the BCR. CD19 acts as a critical co-receptor for signal transduction and it starts a cascade after phosphorylation. The function of CD81 is unknown, but in the absence of expression, CD19 expression is halved and it is thought that it provides docking sites for various signal transduction pathway molecules.[3,4] Co-engagement of the BCR and the co-receptor complex has been shown to reduce the threshold of B-cell activation.[5]

Over the last decade, a few patients have been defined with deficiencies of CD19,[6,7] CD21,[8] and CD81[4] in the literature accompanying changes in B-cell pool, poor immune response to antigens, and so on. Lymphocyte phenotypes may show variations due to the influence of gender, age, ethnicity, or lifestyle differences. The objective of this study was mainly to establish national and regional reference values for BCR co-receptors (CD19, CD21, and CD81) and also other B-cell surface markers, CD20, CD22, and CD27, in healthy Turkish pediatric population.

Patients and methods

Study design and population

The study was conducted at Ege University Faculty of Medicine, Department of Pediatric Immunology. Blood samples were collected from children who were healthy in appearance; aged between 0 and 18 years with normal levels of blood pressure, pulse rate, and hemoglobin; a proportionate height–weight ratio; and no cough/fever/infection or no past history of immune or autoimmune disease and medication. All children underwent a clinical evaluation and the demographic information including name, gender, date of birth, family history, consanguinity, and laboratory data were recorded using a questionnaire. The study group (n=90) was evaluated as three subgroups on the basis of age: 0–1 year (n=30), 1–6 years (n=30), and >6 years (n=30). The study was approved by the ethics committee of Ege University and written informed consent was obtained from all parents.

Laboratory evaluation

All blood samples were collected in ethylenediaminetetraacetic acid (EDTA) and anti-coagulant-free tubes and were screened within 2 h of storage at room temperature.

Whole blood count, absolute lymphocyte counts, relative ratio, and hemoglobin analyses were performed with hemocounter (Cell-Dyn 3700; Abbott Diagnostics, USA).

All flow cytometric analyses were made by using FacsCalibur (Becton Dickinson, BD, USA) equipped with two lasers (488 and 635 nm) and four fluorescence detectors. The data in the text were given according to standardized publishing rules. A total of 10 µL of each monoclonal antibody (BD Biosciences, USA) was added to 100 µL of prepared whole blood in the test tubes. After vortexing, tubes were incubated for 20 min at room temperature in darkness. A total of 2 mL of lysing solution (BD Biosciences) was added, and the mixture was vortexed and incubated for 10 min at room temperature in darkness. The mixture was centrifuged for 1 min and the supernatant was aspirated. Totally, 2 mL of phosphate-buffered saline (PBS) was added to resuspend the pellet and was centrifuged for 1 min and supernatant was aspirated again. Next, 500 µL of staining buffer was added and then cells were analyzed.

The multicolor antibody reagents used for staining were as follows: CD3 FITC/CD4 APC/CD8 PE/CD45 PERCP (clones SK3/2D1/SK7/SK1), CD3 FITC/CD19 APC/CD16-56 PE/CD45 PERCP (clones SJ25C1/2D1/SK7-B73.1/NCAM16.2), CD3 FITC/HLA-DR PE (clones SK7/L243), CD19 PERCP-CY5.5 (clone SJ25CI), CD20 FITC (clone L27), CD21 PE (clone B-ly4), CD22 PE (clone S-HCL-1), CD27 FITC (clone M-T271), and CD81 APC (clone J8-81). The percentages and absolute counts of lymphocyte subsets (CD3$^+$ T cells, CD19$^+$ B cells, CD3$^+$CD4$^+$ T helper cells, CD3$^+$CD8$^+$ T cytotoxic cells, CD3$^+$HLA$^-$DR$^+$ active T cells, CD3$^-$CD16$^+$CD56$^+$ natural killer cells and CD20$^+$, CD19$^+$CD20$^+$, CD21$^+$, CD19$^+$CD21$^+$, CD19$^+$CD21$^{-/low}$, CD22$^+$, CD19$^+$CD22$^+$, CD19$^+$CD27$^+$, CD19$^+$CD27$^+$IgM$^+$, CD19$^+$CD27$^+$IgM$^-$, CD81$^+$, CD19$^+$CD81$^+$ cells) were all investigated. Lymphocytes were gated based on their forward and side scatters and all cell subpopulations were acquired using Cell Ouest-Pro software (BD Biosciences). A sample of gating strategy is explained in Figure 1. The total lymphocyte population was identified on the basis of forward (FSC) and side (SSC) scatter characteristics. B cells were defined as CD19 expressing cells in the lymphocyte population.
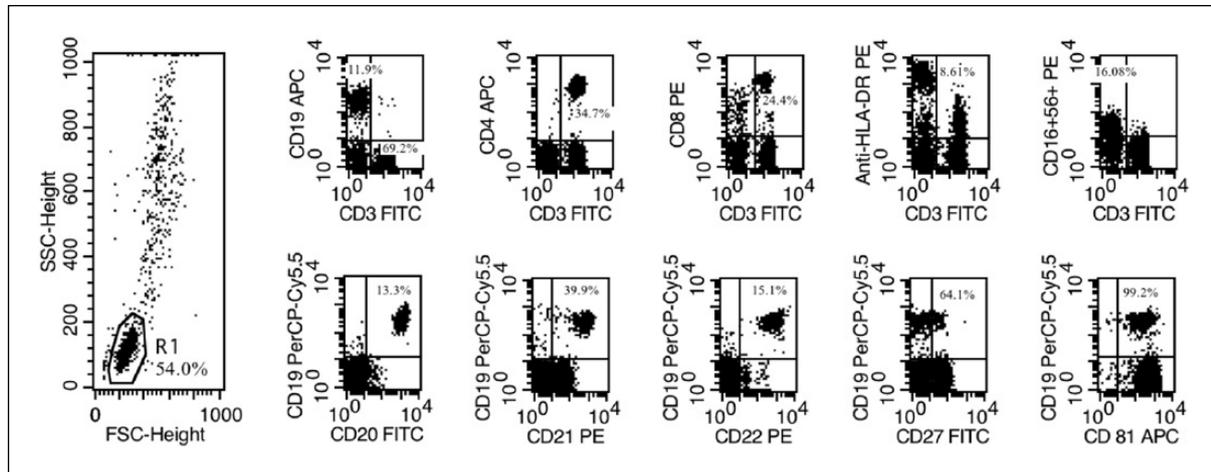
Figure 1. An example for the gating strategy of cell surface markers (R1: total lymphocyte gate).

Statistics

Statistical analyses were performed by using SPSS Windows Version 16.0 (SPSS Inc., USA). One sample Kolmogorov–Smirnov test was used to check the Gaussian distribution of all variables and normal distribution was seen. The mean, standard deviation (SD), and 2.5%–97.5% percentile values were calculated for all parameters. One-way analysis of variance (ANOVA) was used to check for significant differences between age groups. A two-sided P value <0.05 was considered as statistically significant.

Results

A total of 90 healthy Turkish children were recruited for study including 34 females (37.8%) and 56 males (62.2%) with a mean age of 60.5 ± 61.1 (range: 1–282) months. The children were categorized as three equally distributed (n=30) subgroups according to age: 0–1 year (1–12 months old; 9 girls, 21 boys; mean age: 6.80 ± 3.57 months); 1–6 years (16–72 months old; 15 girls, 15 boys; mean age: 42.0 ± 14.7 months), and >6 years (76–282 months old; 10 girls, 20 boys; mean age: 132.6 ± 50.3 months).

Demographical and general laboratory data of 90 healthy Turkish children are given in Table 1. The absolute leukocyte and lymphocyte counts decreased from the 0- to 1-year group throughout adolescence. The relative size and absolute counts (mean±SD, 2.5%–97.5% percentile) of lymphocyte subsets and surface markers are listed in Tables 1 and 2.

As shown in Table 1, the absolute counts of $CD3^+$ T lymphocyte were higher in the first year of life and decreased with age by half, but the relative frequency of T cells remained stable. The absolute number of $CD3^+CD4^+$ T lymphocytes decreased threefold from 0–1 year to >6 years age group, but this reduction was not proportional to the decrease in relative size. The mean percentage of $CD3^+CD8^+$ T cells increased from 19.6% (0–1 year) to 25.7% (>6 years). The absolute count of $CD3^+CD8^+$ T cells was halved from 0–1 year to >6 years. The $CD19^+$ B-cell absolute counts were initially higher during the first year of life and then decreased to one-third in the following years. The relative size increased after the first year and decreased to adult values after 6 years of age. Natural killer (NK) cell frequency remained stable until 1 year, to subsequently increased by half, while their absolute counts showed unrelated changes. The mean relative frequency of $CD3^+HLA$-DR^+ active T cells increased with age, changing from 6.57% to 8.17% in adolescence. All lymphocyte subsets showed statistically significant difference between the categorized age groups (Table 1).

The percentage of $CD19^+CD20^+$ B lymphocytes decreased from 21% to 13.6% during growth. $CD19^+CD21^+$ and $CD19^+CD22^+$ % B-lymphocyte percentages were also steadily decreased from birth to adolescence. $CD19^+CD27^+$ memory B-cell percentages increased from 1.21% to 2.41% after 1 year of age and then stabilized. $CD19^+CD27^+IgM^+$ memory B-cell percentages also increased twofold from 5.79% to 10.2%. $CD19^+CD81^+$ B-lymphocyte percentages decreased during growth, while total CD81% remained stable (Table 2).

Table 1. Demographical and general laboratory data and reference ranges of lymphocyte subsets (in total lymphocyte gate) for different age groups in healthy Turkish children.

	0–1 year (n = 30)	1–6 years (n = 30)	>6 years (n = 30)	P
Age (months)				
Mean ± SD	6.80 ± 3.57	42 ± 14.7	132.6 ± 50.3	<0.001
2.5%–97.5%[a]	1–12	16–72	76–282	
Gender (n)				
Female	9	15	10	–
Male	21	15	20	
WBC (cells/mm³)				
Mean ± SD	10,700 ± 3262	9851 ± 2772	7263 ± 2424	<0.001
2.5%–97.5%	3770–16,900	5460–18,300	3690–13,300	
Absolute lymphocyte count (cells/mm³)				
Mean ± SD	6156 ± 2046	4555 ± 1517	2779 ± 921	<0.001
2.5%–97.5%	2370–11,000	1050–9030	1340–5170	
CD3⁺ T cells (cells/mm³)				
Mean ± SD	4411 ± 1480	3220 ± 1180	1989 ± 709	<0.001
2.5%–97.5%[a]	1730–7680	506–7267	804–3837	
CD3⁺ T cells (%)				
Mean ± SD	71.5 ± 8.73	70.0 ± 7.18	71.6 ± 9.51	0.724
2.5%–97.5%	54.2–92.9	48.2–81.4	48.6–89.4	
CD19⁺ B cells (cells/mm³)				
Mean ± SD	1179 ± 583	739 ± 329	377 ± 202	<0.001
2.5%–97.5%	63.9–2151	242–1459	89.1–1067	
CD19⁺ B cells (%)				
Mean ± SD	21.5 ± 6.68	16.5 ± 5.70	13.3 ± 4.78	0.001
2.5%–97.5%	10.5–40.6	6.74–30.4	4.98–26.3	
CD3⁺CD4⁺ T helper cells (cells/mm³)				
Mean ± SD	2217 ± 845	1314 ± 542	818 ± 395	<0.001
2.5%–97.5%	630–3601	118–3245	202–1899	
CD3⁺CD4⁺ T helper cells (%)				
Mean ± SD	49.7 ± 8.54	40.3 ± 7.27	40.0 ± 10.1	<0.001
2.5%–97.5%	30.6–61.3	23.2–59.5	25–62.9	
CD3⁺CD8⁺ T cytotoxic cells (cells/mm³)				
Mean ± SD	902.6 ± 475	803 ± 417	515 ± 244	0.001
2.5%–97.5%	276–2093	108–2367	212–1389	
CD3⁺CD8⁺ T cytotoxic cells (%)				
Mean ± SD	19.6 ± 5.89	24.2 ± 5.48	25.7 ± 5.58	<0.001
2.5%–97.5%	10.2–33.2	15.2–39	15.3–40.9	
CD3⁻CD16⁺-56⁺ natural killer cells (cells/mm³)				
Mean ± SD	344 ± 261	509 ± 295	264 ± 161	0.001
2.5%–97.5%	74.0–1063	143–1599	50–721	
CD3⁻CD16⁺-56⁺ Natural killer cells (%)				
Mean ± SD	5.61 ± 3.07	11.2 ± 4.85	10.0 ± 5.63	<0.001
2.5%–97.5%	1.36–15.1	3.41–26.4	1.47–24.8	
CD3⁺HLA-DR⁺ active T cells (cells/mm³)				
Mean ± SD	388 ± 306	375 ± 235	241 ± 185	0.043
2.5%–97.5%	92–1379	22–954	34–680	
CD3⁺HLA-DR⁺ active T cells (%)				
Mean ± SD	6.57 ± 5.50	7.84 ± 3.70	8.17 ± 5.0	0.408
2.5%–97.5%	2.13–25.2	2.14–16.2	1.34–20.2	

WBC: white blood cells; SD: standard deviation.
[a]Values represent percentile.

Table 2. The frequencies of B-cell co-receptors (CD19, CD21, CD81) and some surface markers (CD20, CD22, CD27) in healthy Turkish children (in lymphocyte gate).

	0–1 year (n = 30)	1–6 years (n = 30)	>6 years (n = 30)	P
CD19+ (%)				
Mean ± SD	21.5 ± 6.68	16.5 ± 5.70	13.3 ± 4.78	0.001
2.5%–97.5%	10.5–40.6	6.74–30.4	4.98–26.3	
CD20+ (%)				
Mean ± SD	22.1 ± 8.61	17.9 ± 5.58	15.8 ± 4.49	0.001
2.5%–97.5%[a]	5.16–42.4	6.84–31.7	8.20–27.0	
CD19+CD20+ (%)				
Mean ± SD	21.0 ± 8.88	16.5 ± 5.38	13.6 ± 4.52	<0.001
2.5%–97.5%	2.47–41.0	6.13–30.9	6.53–23.9	
CD21+ (%)				
Mean ± SD	71.0 ± 7.68	53.0 ± 9.74	39.6 ± 10.6	<0.001
2.5%–97.5%	50.0–85.0	38.1–78.0	22.6–59.9	
CD19+CD21+ (%)				
Mean ± SD	20.7 ± 8.63	16.3 ± 5.31	13.5 ± 4.52	<0.001
2.5%–97.5%	3.05–40.1	6.0–31.2	6.51–23.5	
CD19+CD21−/low (%)				
Mean ± SD	0.65 ± 0.41	0.60 ± 0.30	0.63 ± 0.43	0.884
2.5%–97.5%	0.09–1.82	0.11–1.31	0.09–2.25	
CD22+ (%)				
Mean ± SD	22.0 ± 8.56	18.1 ± 6.21	14.8 ± 3.91	<0.001
2.5%–97.5%	4.71–42.4	7.14–34.9	7.63–21.4	
CD19+CD22+ (%)				
Mean ± SD	20.6 ± 8.66	16.3 ± 5.76	13.1 ± 4.07	<0.001
2.5%–97.5%	2.35–40.7	6.30–30.7	5.71–20.3	
CD19+CD27+ (%)				
Mean ± SD	1.21 ± 0.60	2.41 ± 1.35	2.64 ± 1.08	<0.001
2.5%–97.5%	0.31–2.61	0.84–6.58	1.08 - 4.62	
CD19+CD27+IgM+ (%)				
Mean ± SD	5.79 ± 6.29	10.2 ± 3.70	12.9 ± 6.74	<0.001
2.5%–97.5%	0.22–30.6	4.65–17.5	4.98–27.6	
CD19+CD27+IgM− (%)				
Mean ± SD	1.55 ± 1.06	9.20 ± 5.15	12.1 ± 4.22	<0.001
2.5%–97.5%	0.08–4.61	3.66–18.8	5.94–20.0	
CD81+ (%)				
Mean ± SD	99.6 ± 0.26	99.4 ± 0.45	99.0 ± 0.65	<0.001
2.5%–97.5%	98.8–99.9	97.6–99.9	96.4–99.9	
CD19+CD81+ (%)				
Mean ± SD	20.7 ± 8.68	16.0 ± 5.16	13.4 ± 4.53	<0.001
2.5%–97.5%	2.41–40.9	6.81–25.8	6.14–25.2	

SD: standard deviation.
[a]Values represent percentile.

Discussion

The most obvious changes in the composition of the peripheral B-cell pool occur in the first 5 years of life.[1] In this study, common lymphocyte subset frequencies were in accordance with the previously published data[9] (Table 1). CD19+ B-cell absolute counts were initially higher during the first year, and then decreased to one-third. Moraes-Pinto et al.[9] had observed an increase in absolute counts during the first 2 years with a subsequent reduction. CD21 which interacts with CD19 to generate transmembrane signals and instructs B cells toward inflammatory responses is expected having parallel trends with CD19 or CD81 throughout life and CD21 deficiency causes a moderate hypogammaglobulinemia.[8] CD19+CD21+ lymphocyte percentages were steadily decreased from birth to adolescence in our study group (Table 2). The frequency of CD21−/low B cells in peripheral blood is mainly composed of memory B

cells[10] and absolute counts increase during first 5 months, then gradually decreases and it is often expanded in autoimmunity and immunodeficiencies. CD19+CD21−/low cells showed no difference between age groups. CD81, which is required for efficient collaboration between the B-cell receptor and CD21, CD19, and various signaling enzymes, is also required for maturation and surface expression of CD19. CD81 deficiency alone results in a more prominent decrease in transitional cells and CD19/CD81 deficiency reduces memory B cells accompanying skin and gastrointestinal infections.[4] CD19+CD81+ B cells decreased during growth, while total CD81% remained stable which might be explained by wide expression on other immune cells. Mature B-cell-specific molecule CD20 deficiency results in impaired T-cell independent antibody responses, reduction in memory B cells and response to pneumococcal polysaccharides.[11] The total CD20+ and CD19+CD20+ B-cell frequencies were steadily decreased from birth to adolescence in parallel to total B lymphocytes (Table 2). In a previous study,[12] total CD20+ B cells had increased similar to lymphocytes from cord blood to infancy, and then gradually had decreased through 10–18 years of age. CD22 regulates follicular B-cell survival and negatively regulates B-cell signaling.[13] CD19+CD22+ B cells showed a decrease similar to total B cells indicating maturation. CD27, which regulates T, B, NK, and plasma cell function, is also a marker for memory B cells. CD27 deficiency has been published for persistent symptomatic Epstein–Barr virus (EBV) viremia with hypogammaglobulinemia, impaired T-cell-dependent antibody generation,[14] and also immune dysregulation syndrome. Morbach et al.[1] suggested that the decrease in total B-cell count within the first 5 years of age was related to the reduction in transitional and naive B cells due to decreased bone marrow output. After first year, the absolute B-cell counts remained stable while the shift from naive to memory B cell was continued. CD19+CD27+ memory B cells increased twofold similar to Duchamp et al.'s[15] findings and remained stable for the rest of adolescence ages.

This study establishes new reference values for B-cell co-receptors (CD19, CD21, CD81) and surface markers (CD20, CD22, CD27) in healthy Turkish children which may be used by the research community. A clinician can compare his or her own data such as CD21 or CD81 levels with the reference values and if the result is lower than mean − 2SD of reference value, the patient may have a possible diagnosis of deficiency of this lymphocyte surface marker. These pediatric reference intervals will be useful for the design of the new studies for the evaluation of diagnostic or classification criteria of primary immunodeficiencies in future. And also, the normal values will be helpful for possible diagnosis of B-cell abnormalities and common variable immune deficiency (CVID) patients with hypogammaglobulinemia before performing molecular and genetic studies or if the clinical findings also match with the deficiency, the patient may have an exact diagnosis by having molecular analysis of only "suspected genes." In other words, there will be no need for whole exome sequencing and this will be more economic and time saving.

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